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Evaluation of a new compound for the treatment of arthritis in the AIA rat model

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Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease, which mainly affects the joints. RA is characterized by pronounced joint swelling, with progressive destruction of articular cartilage and bone, leading to functional impairment and increased morbidity and mortality. It is very important to have an early and accurate diagnose and treatment in order to prevent severe articular lesions. The pathogenesis of RA involves several immune cells and complex cytokine networks which perpetuate the inflammatory process and promote bone resorption and damage. Despite the existence of several drugs available for the treatment of RA, disease remission is still an unmet medical need for the great majority of patients.

X, a Chinese herbal compound, has significant anti-inflammatory properties in some auto-inflammatory diseases, namely colitis and multiple sclerosis as well as in cancer. Our lab has previously shown significant anti-inflammatory effects of X in an adjuvant-induced arthritis (AIA) rat model.

In the present work we hypothesized that X is a promising candidate for the safe treatment of inflammation and bone damage in arthritis. Thus, our main objective was to evaluate the anti-inflammatory and bone protective effects of X *in vivo* using the AIA rat model of arthritis. We treated AIA rats with X intraperitoneally at a dose of 1µg/g daily, in two different groups of treatment: one group initiated X treatment in the early phase of arthritis (4 days after disease induction) and the other group initiated the treatment in a later phase of disease progression (11 days after disease induction). After 22 days of disease duration, animals were sacrificed and samples were collected to access X efficacy, toxicity, anti-inflammatory and bone protective properties, locally (joint articular tissues) and systemically (serum).

Our results have shown that X treatment was able to safely diminish the inflammatory signs, even when treatment was initiated in a later phase of arthritis progression. X was able to decrease the systemic levels of IL-6 and bone turnover markers. Moreover, locally, X treatment decreased the infiltration and proliferation of immune cells as well as bone erosions in the joints of treated animals. Bone mechanical properties were recovered in X early-treated rats.

In conclusion, this work gives an insight in the synovial homeostasis and bone protective properties of X treatment in the AIA rat model of arthritis. Future work will focus in X bone quality protection. Altogether, we expect that our results give support to a pre-clinical trial.

Keywords: Rheumatoid arthritis, X, adjuvant-induced rat model of arthritis, inflammation, bone turnover.

Resumo

A artrite reumatóide (AR) é uma doença crónica inflamatória autoimune, que afeta principalmente a membrana sinovial das pequenas articulações e que pode levar à destruição da cartilagem e osso subjacentes, causando um elevado grau de incapacidade, morbilidade e redução da esperança média de vida nos doentes. Numa fase inicial, manifesta-se através de inchaço, rigidez e dor nas pequenas articulações que progressivamente se alastra para as articulações maiores. É de salientar a importância de um diagnóstico e tratamento precoce e adequado, de modo prevenir o aparecimento de lesões mais graves.

No processo inflamatório característico da AR estão envolvidos tanto o sistema imunitário inato como adaptativo, que atuam num processo contínuo de produção de citocinas e de recrutamento de células inflamatórias para a membrana sinovial, que reveste as articulações, tornando-a hiperplásica. Células T auto-ativadas desencadeiam a ativação de monócitos, macrófagos e fibroblastos, que por sua vez produzem citocinas pró-inflamatórias que perpetuam o sinal inflamatório, mediadores de atividade óssea e enzimas com capacidade para reabsover cartilagem e osso.

O processo de remodelação óssea envolve a destruição e formação de osso, de uma forma controlada e que envolve vários tipos celulares: osteoclastos, osteoblastos e osteócitos. Existem também vários mediadores celulares, nomeadamente RANKL e OPG, que induzem e inibem a formação ou destruição de osso. Alguns dos mediadores e produtos destes processos podem servir como marcadores de *turnover* ósseo e da cartilagem, permitindo perceber o balanço da remodelação óssea.

Apesar de ainda não existir cura para a AR, são já aplicadas várias abordagens terapêuticas, que incluem numa fase inicial de tratamento os glucocorticoides, que por terem efeitos secundários adversos em doses elevadas, são substituídos, numa segunda fase por DMARD's (agentes antirreumáticos modificadores de doença), que incluem o metotrexato, o fármaco mais usado no tratamento destes doentes. No entanto, uma grande percentagem de doentes é não-respondedora ou deixa de responder ao tratamento, tendo por isso surgido uma nova categoria de fármacos, os DMARD's biológicos, que são geralmente anticorpos que atuam sobre, por exemplo, TNF, IL-1, células B e T. Para alguns doentes, nenhuma destas terapias é eficaz, sendo por isso necessário continuar a investir na descoberta de novo medicamentos com melhores resultados e poucos efeitos secundários.

O X, um composto extraído da planta *Y*, muito usado na medicina chinesa, mostrou ter efeitos anti-inflamatórios em várias doenças autoimunes, como a colite crónica a esclerose múltipla, e também no cancro. O nosso laboratório reportou também

que este composto tem propriedades anti-inflamatórias num modelo animal de artrite (AIA) em rato.

No presente trabalho o objetivo principal foi testar o X no tratamento da artrite, usando um modelo de artrite AIA. As suas propriedades anti-inflamatórias, anti-proliferativas e protetoras do osso foram avaliadas.

Os animais foram tratados intraperitonealmente com X (1µg/g diariamente) e divididos em dois grupos, com diferentes dias de início da administração: um dos grupos iniciou o tratamento ao dia 4 e outro iniciou o tratamento ao dia 11 após a indução da doença. Ao fim de 22 dias de duração da doença os animais foram eutanaziados e foram colhidas amostras de soro, órgãos (fígado, rim e baço) e ossos (fémures, tíbias e vértebras).

A avaliação do score inflamatório e do diâmetro da pata dos animais permitiram perceber que a administração de X foi significativamente eficaz no tratamento dos sinais inflamatórios da artrite, tanto no grupo de animais que iniciou o tratamento numa fase inicial do desenvolvimento da doença como no grupo que começou o tratamento numa fase mais tardia. O peso, parâmetros bioquímicos de toxicidade e a histologia dos órgãos indicaram também que a administração de X intraperitonealmente nesta concentração não causou quaisquer efeitos secundários nos animais.

Do painel de citocinas avaliadas (IL-1β, IL-6, IL-17 e TNF) apenas a citocina IL-6 se observou significativamente aumentada no soro dos animais doentes em relação aos animais saudáveis. O tratamento com X diminuiu significativamente os níveis de IL-6 em ambos os grupos de tratamento, comparativamente aos animais não-tratados. Foram ainda analisados no soro mediadores pro-inflamatórios envolvidos na osteoclastogénese, OPG e RANKL, e indicadores de atividade óssea, CTX-I, P1NP e TRACP-5b, e da cartilagem, CTX-II. Não se encontraram diferenças significativas no rácio RANKL/OPG entre animais saudáveis e doentes não-tratados. No entanto, foram observados níveis aumentados de CTX-I, P1NP e CTX-II nos animais doentes, em comparação com os animais saudáveis. O tratamento com X diminuiu significativamente os níveis de P1NP, CTX-II e TRACP-5b em ambos os grupos de tratamento, em relação aos animais doentes não-tratados. Não se encontraram diferenças entre os grupos de tratamento e os animais doentes não-tratados para o CTX-I.

Realizou-se também uma avaliação histológica das patas dos animais tendo em conta os níveis de infiltração e proliferação da membrana sinovial, erosão óssea e impacto global da doença na estrutura articular. Observou-se que ambos os animais em ambos os grupos de tratamento com X têm níveis de infiltração e proliferação da membrana sinovial significativamente diminuídos em relação aos animais não-tratados, apresentando um fenótipo semelhante aos animais saudáveis. Observou-se também

que as erosões ósseas aparecem desde uma fase muito inicial do desenvolvimento da doença, e que o tratamento com X é capaz de reduzir significativamente o aparecimento de novas erosões ósseas na articulação. As patas foram ainda analisadas por imunohistoquímica, usando marcadores de células imunes inflamatórias. Os animais tratados com X tinham significativamente menos células sinoviais em proliferação e uma redução do número de células T, B e macrófagos presentes na membrana sinovial, quando comparados com os animais doentes não-tratados.

As propriedades mecânicas do osso foram avaliadas através de 3-point bending nos fémures dos animais. Verificou-se que os animais não-tratados têm uma menor fase elástica e plástica em comparação com os animais saudáveis, sendo que o tratamento com X foi capaz de preservar as propriedades mecânicas do osso no grupo de animais que começou o tratamento no dia 4. Os animais com início do tratamento ao dia 11 após indução da artrite não conseguiram recuperar estas propriedades mecânicas ósseas.

Estes resultados apontam o X como uma terapêutica promissora para o tratamento da artrite reumatoide dadas as suas características anti-inflamatórias e protetoras do dano ósseo.

Palavras-chave: Artrite reumatóide, X, modelo de artrite induzida por adjuvante em rato, inflamação, atividade óssea.

Abbreviations

APC – antigen presenting cell

ACPA – anti-citrullinated protein antibody

ACR – american college of rheumatology

AIA – adjuvant-induced arthritis

BMD – bone mineral density

CIA – collagen-induced arthritis

CRP – c-reactive protein

CTX – c-terminal telopeptides

DMARD – disease-modifying anti-rheumatic drug

ESR – erythrocyte sedimentation rate

EULAR – european league against rheumatism

HLA – human leukocyte antigen

IFNγ – interferon gamma

IL – interleukin

MHC – major histocompatibility complex

MMP – metalloproteinase

NFATC1 – nuclear factor of activated T-cells-1

NF-kB – nuclear factor kappa-light-chain-enhancer of activated B cells

OPG – osteoprotegerin

Osx – osterix

P1NP – N-terminal propeptide of type 1 procollagen

RA – rheumatoid arthritis

RANK – receptor activator of nuclear factor NF-kB

RANKL – RANK ligand

RF – rheumatoid factor

Runx 2 – runt-related transcription factor 2

TCR – T-cell receptor

TLR – toll-like receptor

TNF – tumor necrosis factor

TRACP – tartrate resistant acid phosphatase

Introduction

RHEUMATOID ARTHRITIS

Definition

Rheumatoid arthritis (RA) is a chronic, progressive, autoimmune inflammatory disease, mainly characterized by synovial inflammation, cartilage and bone destruction[1,2]. This disease is often associated with other comorbidities, such as cardiovascular, pulmonary, psychological and skeletal disorders[3]. If not properly treated, RA causes considerable personal and economic costs as it is associated with serious disability, reduced quality of life, loss of work capacity and reduced life expectancy[4]. Epidemiological studies have shown that RA affects about 0.5-1.1% of adult population worldwide, is more frequent in women than men (ratio 3:1) and disease onset occurs around the age of 50 years-old[5].

The predominant symptoms of the disease are symmetrical inflammation of small joints (hand and feet), swelling, stiffnes and pain with the gradual involvement of larger joints[6]. RA diagnosis is performed according to the 2010 American College of Rheumatology/European League Against Rheumatism (2010 ACR/EULAR) criteria. This criteria takes into account the clinical history of the patient, the number and size of the affected joints, the presence of rheumatoid factor (RF) and anti-citrullinated protein antibody (ACPA), the levels of inflammatory markers in the blood, such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), radiologic analyses and symptoms duration[7]. It is important to diagnose RA in the earliest possible phase of the disease course, since a prompt diagnosis and an accurate early therapeutic strategy are essential to prevent disease progression and joint erosions[8].

Etiology

Although the etiopathogenesis of RA is not yet completely understood, the most plausible explanation is a combination of genetics and environmental factors[9]. Human Leukocyte Antigen (HLA) is the most important genetic factor for RA susceptibility. HLA gene encodes HLA class II molecules that are responsible for the antigen presentation to T cells, by presenting antigens to T-cell receptors (TCR), leading to T cell activation. Certain haplotypes, with a specific sequence may lead to an enhanced stimulation and expansion of autoantigen-specific T-cells in the joints[10]. The three more common alleles in the Caucasian population are: HLA-DRB1*01, HLA-DRB1*04 and HLA-DRB1*10[11]. Regarding environmental risk factors, smoking is largely the most established one.

Studies of gene-environmental factors interaction have shown that cigarette smoking has been associated with an increased risk of developing RA in individuals that express HLA-DRB1 allele[12]. In addition, age, gender, infectious agents, hormonal and dietary factors are also associated with the occurrence of this disease[5].

Pathophysiology

The synovial joint is composed of two bony ends covered with cartilage and separated by a synovial space, involving the synovial fluid and membrane. In healthy individuals, the synovial membrane is almost an acellular structure, with a thin layer of synoviocytes (mainly macrophage and fibroblast-derived cells), covering and protecting bone ends. In the case of RA patients, the synovial space is composed of a variety of mononuclear cells, such as T cells, B cells, plasma cells, neutrophils, macrophages and mast cells that infiltrate the synovia, which becomes hyperplasic[13,14]. This phenomenon is not only caused by the homing of these cells into the synovia, but also by the perpetuation of the inflammatory process due to the production of cytokines that maintain inflammatory cells activated in a positive feedback loop[15]. Therefore, synovial inflammation characteristic of RA involves a complex network of immune cells and cytokines (Figure 1). The activation of invading T cells, mediated by the binding of their TCR to (auto)-antigen MHC on antigen presenting cells (APCs), leads to the activation of synovial monocytes, macrophages and fibroblast, through the production of interferon-γ[16,17]. This will lead to overproduction of pro-inflammatory cytokines, such as interleukin (IL)-1[18], IL-6[19], IL-17[20] and tumor necrosis factor (TNF)[21], the major contributors to the chronic inflammatory process[22]. IL-1, IL-17 and TNF will induce macrophages to expresse the receptor activator of nuclear factor-κB (RANK), which after binding to its ligand (RANKL), stimulate their differentiation into mature osteoclasts, able to resorb and destroy adjacent bone[23]. In RA patients, cartilage, which protects the bone ends, is also destroyed by the action of metalloproteinases (MMPs), produced by synovial activated cells[2,14]. Altogether, the infiltration of immune cells and the proliferation of joint lining synovial fibroblasts lead to the formation of a tumor-like tissue, the pannus, which invades and destroys the underlying joint cartilage and bone, resulting in irreversible damage of the bone and in the loss of the normal joint structure, causing functional disability[24].

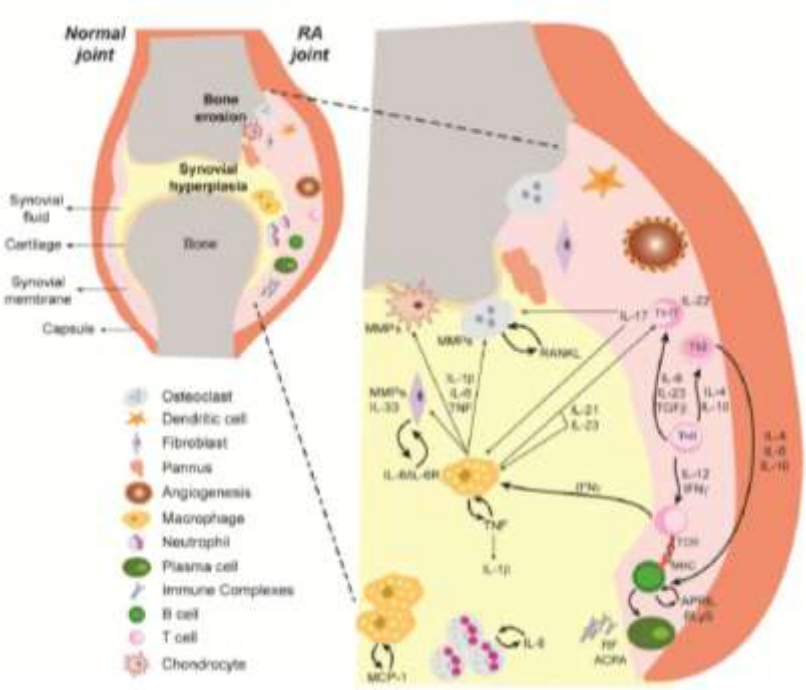

Figure 1- Immune cells and cytokine networks in rheumatoid joints *(adapted from 94)*

Bone homeostasis and RA

Bone homeostasis is highly affected by RA. In healthy individuals, bone formation and resorption are two balanced processes[25]. Bone is a dynamic connective tissue composed of two phases: a mineral phase of inorganic particles of hydroxyapatite embedded in an organic matrix mainly composed of type 1 collagen fibers. This composition and organization of bone matrix results in unique mechanical properties such as stiffness, rigidity, ductility and tensile strength[26]. Osteoblasts are responsible for bone formation. These are cuboid-shaped cells, lining the bone matrix, which are responsible for the secretion of collagen-I and other proteins, such as osteocalcin and osteopotin. Osteoblasts are cells derived from the myeloid lineage, through the expression of Osterix (Osx) and Runx 2[27].P1NP (N-terminal propeptide of type I procollagen) is a product released during bone formation, and used as a serum marker of this process[28,29]. During the mineralization process, some osteoblasts get trapped inside the bone matrix. These cells become the non-proliferative and fully differentiated osteocytes, acting as mechanosensors. These two types of cells, are not only responsible for bone formation and mechanic sensing, but also have an important role on the regulation of osteoclast activity[30,31]. Osteoclasts are multinucleated cells that are able to resorb bone matrix. These cells are derived from the fusion of macrophages in a process named osteoclastogenesis, which requires the expression of RANK and NFATC1 by osteoclast precursors. The binding of RANKL (produced by osteoblasts) to

RANK leads to the fusion of these precursors into mature osteoclasts. Mature osteoclasts are able to resorb adjacent bone, in a process that involves polarization, acidification of the resorbing lacunae and the secretion of proteolytic enzymes, such as cathepsin-K and metalloproteinases[32,33]. This process releases in circulation CTX-I and II (C-terminal telopeptides-I and II, products of collagen type-I and II degradation)[34], and these products are used as serum markers of bone and cartilage destruction, respectively. Between osteoblasts and osteoclasts exists a bidirectional signaling, allowing a tight regulation of bone homeostasis. For example, osteoblasts secrete osteoprotegerin (OPG) that recognizes soluble RANKL, and acts as its decoy receptor, preventing osteoclastogenesis[35].

Bone turnover can be evaluated by measuring serum levels of bone and cartilage markers[36]. In RA patients, it has been shown by Corbet et al[37] that P1NP and CTX-I were significantly elevated in the serum and correlated with femoral neck BMD loss. Moreover, it has been demonstrated that TRACP-5b is associated with disease duration and severity, acting as a good biomarker for bone resorption and osteoclast number[38–40].

Treatment options

The presentation and the clinical course of RA patients as well as the response to the available treatment options are highly variable. The strategy of disease treatment relies on an early diagnosis and prompt adequate treatment with the goal of inducing remission in order to preserve joint function and maintain the quality of life. RA treatments options can be divided in the following categories: glucocorticoids, conventional disease-modifying anti-rheumatic drugs (DMARDs) and biologic DMARDs. Glucocorticoids are used in an initial phase of the disease and are useful to control symptoms in the first weeks of disease diagnosis, until slower-action DMARDs can start to have an effect. Glucocorticoids can have an effect in limiting joint damage, but long term adverse effects, particularly at higher doses, limits their usefulness[41]. Conventional DMARDs, which include methotrexate, are the most used drugs to efficiently treat these patients[42]. DMARDs have a relatively slow onset of action (1 up to 6 months) but they are effective on the long term and are considered to have an acceptable safety[42,43] either in monotherapy or in combination therapy. However, around 30% of the patients are either non-responsive to DMARDs or lose their response secondarily or even develop adverse effects that are incompatible with their use[44–46]. Recently, 9 new biological agents, namely TNF, IL-1 and T-cell activators and B-cell targeted therapies[47,48], have appeared as an alternative to conventional DMARDs (Table 1). However, despite these treatment options, many patients fail to respond to these therapeutics and only 30% of RA patients

achieve remission, thus the development of more effective and safer treatments is still a main concern[48].

Table 1 – *Therapeutical options for the treatment of rheumatoid arthritis.(Adapted from [42])*

Category	Example
NSAIDs	Aspirin, Ibuprofen
Glucocorticoids	Prednisone, methylprednisolone
DMARDs	Methotrexate, hydroxychloroquine, sulfasalazine, leflunomide
Biological agents	
Anti TNF	Infliximab, Etanercpt, Adalimumab
IL-1 inhibitors	Anakinra
T-cell activators inhibitors	Abatacept
B-cell targeted therapies	Rituximab

X

The Chinese medicine, known for its herbs, have for a long time used their products in the treatment of some diseases[49]. One of these compounds is X, a tripterine extracted from the root of *Y*, a perineal vine found in the Asian continent[50]. One of the molecular targets of X is the NF-κB pathway, which is involved in inflammatory processes[51]. Several studies have shown that X have potential therapeutic effects in some autoimmune diseases, interfering with several cellular mediators. Zhu et al[52] have shown in a murine model of Crohn's disease that X ameliorates experimental colitis, reducing inflammation and the expression of pro-inflammatory cytokines (IL-1, IL-17, TNF and IFN-γ) in colon tissues of treated mice. Hasby et al[53] reported a systemic decrease in pro-inflammatory cytokines and a local decrease in TLR2 and CD3 expression in brains of X treated rats in a model of multiple sclerosis. Many others have described beneficial effects of X in the treatment of cancer, through the inhibition of cell migration and proliferation as well as metastasis prevention[54–56].

IL-1β plays an important role since the early phase of RA. The levels of IL-1β are increased in the serum of very early RA patients[57] and in the serum and synovial fluid of established RA patients[58]. This cytokine together with TNF are two major players in RA physiopathology, with uncoupled activites in joint swelling and bone/cartilage destruction. Some reports have shown that after anti-TNF treatment there is no reduction in the circulating and synovial levels of IL-1β and that *in vivo* anti-TNF treatment is not sufficient to prevent arthritis progression[59]. Therefore, pathways regulating IL-1β and

TNF, such as NF-κB, might constitute promising combined therapeutic targets. With this rational, our group performed an *in vitro* drug screening for compounds that simultaneously inhibit IL-1β and TNF secretion, and identified X as a potent inhibitor of IL-1β and TNF secretion as well as NF-κB activation[60]. Moreover, our group has previously shown in an adjuvant-induced rat model of arthritis (AIA) that X was able to significantly suppress inflammatory symptoms in arthritic joints[60]. In addition, Moudgil et al[61] reported, in the same animal model, that X therapeutic properties might be due to its capacity to reduce the ratio of effector T cells vs regulator T cells. The same author has also shown that X's anti-arthritic properties include the reduction of pro-inflammatory cytokines, *in vivo*, as well as an inhibition of leukocyte migration, *in vitro*[62]. The authors have described that the decrease in pro-inflammatory cytokine levels is accompanied by the reduced anti-CCP production and metalloproteinase-9 activity (responsible for bone and cartilage matrix degradation)[63]. Moreover, Liu et al, described in cells isolated from RA patients and cultured *in vitro,* that this compound inhibits human RA fibroblast-like synoviocytes migration and invasion possibly through suppression of MMP-9[64], and suppression of CXC chemokine receptor 4 (CXCR4) expression[55], that is involved in invasion processes. In addition to X anti-inflammatory properties, there are already some evidences that X might have effects in protect inflammation-induced bone damage. Tan et al[65], using micro-CT to scan the ankle joint of DBA1 mice with collagen-induced arthritis (CIA), showed that X treatment markedly reduced joint destruction and TRAP (mature osteoclasts marker) positive cells in areas of bone erosion. Mougdil et al[66], revealed that X significantly reduces pannus formation as well as bone and cartilage destruction in the AIA rat model. Extracts from *Y* have been used for years in Chinese patients with RA, but there are few data in the literature[67]. A recently published meta-analysis concludes that despite the fact that most of the studies were methodologically poor, the evidence so far suggests that extracts of *Y*, of which X is a component, reduce disease activity and can be as efficient as conventional DMARDs in the treatment of RA[68]. Other meta-analyses have been published and results regarding efficacy and safety have been contradictory[67,69,70]. Gastrointestinal tolerability of these extracts might be an issue[71] and future studies will have to be adequately powered, better designed and include other population (9 studies were done exclusively in Chinese populations where these extracts are used in traditional medicine). Importantly, plant extracts are heterogeneous products, exhibiting a high degree of variability, difficult to control in terms of quality and compound concentration and the products used for extraction might create tolerability problems. Altogether, these data suggest that a pure bioactive molecule isolated from *Y*, such as X, could be not only effective but also safe in the treatment of RA.

Aims

RA treatment has evolved profoundly over the past decade, but despite the development of new therapeutic options, it remains a progressive and debilitating disease. Therefore, an effective and safe RA treatment is still an unmet medical need. Our previous results from an *in vitro* drug library screening and *in vivo* AIA rat model preliminary tests, showed that X has significant anti-inflammatory and anti-proliferative properties in the treatment of arthritis when intraperitoneally administered[60]. Based on our data and additional evidence from the literature, we hypothesize that X is effective in controlling not only the inflammatory signs but also the systemic and local degradation of bone that occur in RA patients.

The main goal of this project is to test X for the treatment of arthritis by studying it's anti-inflammatory, anti-proliferative and bone protective properties using the AIA rat model of arthritis.

Specific aims:

Aim 1 - To test the intraperitoneal administration of X *in vivo* in the AIA model for the treatment of arthritis;

Aim 2 - To evaluate the toxicological profile of X *in vivo*;

Aim 3 - To study the effect of X in the circulating levels of pro-inflammatory cytokines as well as bone and cartilage turnover markers;

Aim 4 - To evaluate the anti-inflammatory properties of X in joint articular tissues;

Aim 5 - To analyse the effect of X on systemic bone quality.